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(54) METHOD OF MEASURING AMOUNT OF PROTEIN

(57)Abstract:

PROBLEM TO BE SOLVED: To provide a method of measuring the amount of multichannel protein in a short time and by few processes.

SOLUTION: This method of measuring the amount of protein comprises processes of (1) pouring the sample liquid prepared by making tissue or cell soluble into a well in a plate provided with at least one well and a hydrophobic porous film on a bottom part of the well, and sucking the same from a hydrophobic porous film side of the plate to form a solid phase of the protein in the sample on the film, (2) pouring a first antibody having a labeled region or a region reactable with label, and having the specificity to measured protein, into the well to combine the first antibody with the measured protein, (3) eliminating the unreacted first antibody by a washing process, (4) labeling the antibody by making the label react with the region reactable with the label when the unlabeled first antibody is used, (5) measuring the amount of label combined with the measured protein, and (6) calculating the target protein amount by using the

amount of label on the basis of a predetermined standard curve.

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CLAIMS

[Claim(s)]

[Claim 1] The sample solution which was made to solubilize the following process:(1) organizations or cells, and was prepared Have one or more wells and it pours into the well in the plate which has arranged the hydrophobic porosity film at the pars basilaris ossis occipitalis of a well. This film is made to carry out solid phase formation of the protein in a sample by drawing in from the hydrophobic porosity film side of a plate. It pours into inside. (2) -- the 1st antibody which is labeled, has a reactant part with an indicator, and has the protein and singularity for the purpose of measurement -- this -- a well -- When make it combine with the protein for the purpose of measurement, (3) washing processing is performed, the unreacted 1st antibody is removed and the 1st antibody by which (4) labeling is not carried out is used the amount of the indicator which the indicator was made to act on a reactant part with an indicator, and this antibody was labeled, and was combined with the protein for the purpose of (5) measurement -- measuring -- (6) -- the measuring method of the amount of proteins

which consists of computing the amount of purpose proteins by using the amount of this indicator based on the calibration curve produced beforehand.
[Claim 2] The sample which was made to solubilize the following process:(1) organizations or cells, and was prepared Have one or more wells and it pours into the well in the plate which has arranged the hydrophobic porosity film at the pars basilaris ossis occipitalis of a well. This film is made to carry out solid phase formation of the protein in a sample by drawing in from the hydrophobic porosity film side of a plate. It pours into inside. (2a) the 1st antibody which has the protein and singularity for the purpose of measurement -- this -- a well -- Make it combine with the protein for the purpose of measurement, perform (3) washing processing, and the unreacted 1st antibody is removed. It pours into inside. (3a) the 2nd antibody which is labeled, has a reactant part with an indicator, and has this 1st antibody and singularity -- this -- a well -- When the 2nd antibody which is not labeled by making it combine with this 1st antibody (4a) is used the amount of the indicator which the indicator was made to act on a reactant part with an indicator, and this antibody was labeled, and was combined with the protein for the purpose of (5) measurement -- measuring -- (6) -- the measuring method of the amount of proteins which consists of computing the amount of purpose proteins by using the amount of this indicator based on the calibration curve produced beforehand.
[Claim 3] The approach by claims 1 or 2 in which a plate has two or more wells.
[Claim 4] How to measure by the approach by any one of the claims 1-3, and to diagnose a disease based on the obtained amount of proteins.
[Claim 5] The approach of claim 4 that a disease is gastric cancer, colon cancer, a breast cancer, lung cancer, an esophagus cancer, a prostatic cancer, hepatic carcinoma, kidney cancer, vesical cancer, skin carcinoma, a uterine cancer, a brain tumor, a flesh-and-blood kind, or a bone marrow neoplasm.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention is the approach of it being simple and measuring the amount of the minute amount protein of the many items contained in a sample for a short time.
[0002]

[Description of the Prior Art] If it suffers from diseases, such as cancer, in the body of an animal, the phenomenon which the amount of specific minute amount protein decreases, or increases will be seen. Therefore, when a correlation is found out by the class of disease, a critical degree, and the class of protein to fluctuate, it is possible by measuring the amount of the existence of this protein of the inside of the body of an animal to be used for discernment of a disease, or a diagnosis of malignancy. Conventionally, it is known by the quantum of the protein contained at a minute amount in the solubilization liquid obtained from an organization that the ELISA method and western blotting will be used.

[0003] The 1st antibody specifically combined with the protein made into the purpose and the 1st antibody are the approaches it needs two kinds of antibodies with the 2nd antibody similarly combined with the purpose protein specifically although the ELISA method is different. More, if the 1st antibody is fixed on solid phase base materials, such as a well in a microtiter plate, and porous film or a particle, and is made to react with a protein content sample there, the 1st antibody and the protein corresponding to it will combine with a detail. Subsequently, if the 2nd antibody which an indicator is carried out with the enzyme which can carry out assay easily, and has singularity in the purpose protein is made to react further, the 2nd antibody will combine with the protein combined with the 1st antibody. Then, it is the principle that the amount of the protein made into the purpose can be measured, by making the substrate of the enzyme react to the enzyme beforehand labeled by the 2nd antibody, and measuring the amount of the product obtained.

[0004] However, two independent specific antibodies (the 1st antibody and the 2nd antibody) which do not do effect mutually [the binding sites combined with an antigen to one detection purpose protein (antigen) differ, and] are required of the ELISA method as mentioned above. It also takes time amount, in order to perform two immunoreactions again.

[0005] On the other hand, western blotting is an approach of detecting and identifying specific protein, by imprinting on porous film, such as a nitrocellulose membrane and PVDF film, and performing an antigen-antibody reaction on this film, after carrying out fractionation of the sample containing the purpose protein by polyacrylamide gel electrophoresis. More, if the 1st antibody combined specifically is made to react to this protein to the protein imprinted on the nitrocellulose membrane, protein and the 1st antibody will combine with a detail. Subsequently, the enzyme which can carry out assay easily is combined in covalent bond, and if the 1st antibody and the 2nd antibody combined specifically are made to react further, the 2nd antibody will

combine with the protein combined with the 1st antibody. It is the principle that the amount of the protein made into the purpose can be measured, by making the substrate react to the enzyme combined with the 2nd antibody like the ELISA method, detecting existence of protein and measuring the amount of the product by detecting the product obtained.

[0006] Instead of the 2nd antibody which combines the enzyme which can carry out assay easily in covalent bond, and is specifically combined with the 1st antibody, an indicator can be carried out with radioisotope or a fluorescent material, and the 2nd antibody specifically combined with the 1st antibody can also be used. Under the present circumstances, instead of making an enzyme reaction perform to the enzyme beforehand combined with the 2nd antibody, existence of a radioisotope and an amount are measured with autoradiography, or the detection or the amount of protein which measures existence of a fluorescent material and an amount with a fluorescence detection machine, and targets them from the obtained result is computed.

[0007] However, western blotting takes [there is a procedure of imprinting the condition that carried out electrophoresis of the analyte and fractionation was carried out for every molecular weight of the protein on gel, on the porous film, and / each] time amount and is complicated as mentioned above. Moreover, since the amounts of association of SDS differ common protein and a little when using the SDS-polyacrylamide gel by which the surfactant (SDS) of anion nature went into electrophoresis, dependability becomes low for identifying which [of the protein fractionation which the molecular weight obtained by the fractionation on gel may differ from actual molecular weight, therefore was separated] is the purpose protein.

[0008] Furthermore, since whether the band of the protein by which fractionation was carried out by imprinting is imprinted by the concentration of the protein contained in a band in linearity at the porous film cannot guarantee, the band of the imprinted protein lacks in quantum nature. Furthermore, since after electrophoresis needs to perform two steps of antigen-antibody reactions in the protein on the imprinted porous film, its time amount which the whole approach takes is as long as about about ten hours.

[0009] On the other hand, the solid phase enzyme immunoassay (JP,1-223352,A) which is a strange method of the western blotting which fixes directly the purification object of the antigen protein specifically combined with the protein made into the purpose is reported to the porous nitrocellulose membrane. By this approach, the sample containing the target protein is made to react to the antigen protein fixed on

this film, and antigen protein and the purpose protein are combined with it. Subsequently, if the antibody which combined with the united purpose protein specifically, and combined the enzyme which can carry out assay easily in covalent bond is made to react further, an antibody will combine with the purpose protein combined with antigen protein.

[0010] It is the principle that the purpose protein combined with the antibody is detectable, by making the substrate react to the enzyme beforehand combined with the antibody like the ELISA method, and detecting existence of the product obtained. By this approach, since the count of an immunoreaction was reduced compared with the ELISA method or western blotting, an approach is simplified, therefore can be performed in a short time.

[0011] However, although direct protein is fixed on the film by this approach, that protein is the specific antigen of the protein aimed at obtaining a quantum, and is not the protein aimed at obtaining a quantum itself. Therefore, one detection purpose protein and a corresponding specific antigen need the purpose protein and one kind of another corresponding specific antibody further with one kind.

[0012] As mentioned above, the sample containing the protein for the purpose of detection [whether after carrying out fractionation by electrophoresis beforehand or making solid phase fix the specific antibody corresponding to the purpose protein like the ELISA method like western blotting, it is made to react with the 2nd specific antibody, and] Like an approach given in JP,1-223352,A, after making the porous film fix the specific antigen to the purpose protein, as a specific antigen reaction carried out in the 2nd, two or more specific antibodies or an antigen was required to a pretreatment phase and the detection purpose protein.

[0013] Therefore, development of the approach of carrying out direct detection or a quantum was desired in the protein aimed at obtaining [which can perform the quantum of the purpose minute amount protein of many items at few processes in a short time, and is contained to the detection purpose protein during the quantum approach of the protein using one kind of specific antibody, i.e., a sample,] a quantum.

[0014]

[Means for Solving the Problem] According to this invention, the measuring method of the amount of the minute amount protein of many items which can perform directly the sample which the hydrophobic porosity film was made to solubilize an organization or the cell origin, and was prepared at few processes in a short time by carrying out solid phase formation is offered. Since the direct and hydrophobic porosity film is made to specifically carry out solid phase formation of the sample which carried out

solubilization processing of the biopsy specimen extracted from a cell or the whole organization directly according to this invention, purification processing of a complicated sample is unnecessary. Moreover, since one kind of antibody which has singularity is used for the protein for the purpose of measurement according to this invention and the number of the reactions which-izing of the cost which a measuring method takes can be carried out [****], and are performed in the measurement purpose protein can be decreased, the approach of this invention can be performed in a short time. Moreover, since the hydrophobic porosity film is made to carry out or more 1 solid-phase formation of the sample using the plate which has one or more wells according to this invention, the quantum of the protein of many items can be performed about the same sample, and the quantum of the protein of the same item can be performed about many samples.

[0015] The sample solution which was made to solubilize the following process:(1) organizations or cells in a detail, and was prepared in it according to this invention Have one or more wells and it pours into the well in the plate which has arranged the hydrophobic porosity film at the pars basilaris ossis occipitalis of a well. This film is made to carry out solid phase formation of the protein in a sample by drawing in from the hydrophobic porosity film side of a plate. It pours into inside. (2) -- the 1st antibody which is labeled, has a reactant part with an indicator, and has the protein and singularity for the purpose of measurement -- this -- a well -- When make it combine with the protein for the purpose of measurement, (3) washing processing is performed, the unreacted 1st antibody is removed and the 1st antibody by which (4) labeling is not carried out is used the amount of the indicator which the indicator was made to act on a reactant part with an indicator, and this antibody was labeled, and was combined with the protein for the purpose of (5) measurement -- measuring -- (6) -- based on the calibration curve produced beforehand, the amount of this indicator is used and the measuring method of the amount of proteins which consists of computing the amount of purpose proteins is offered.

[0016] Furthermore, this invention offers the approach of diagnosing diseases, such as cancer, based on the result obtained by the measuring method of the amount of proteins of this invention.

[0017]

[Embodiment of the Invention] The approach of this invention can be performed according to the following processes. First, a sample is prepared. A Waring blender and a supersonic wave are used for a sample in the buffer solution containing a surface active agent, a proteolytic enzyme inhibitor, etc., it grinds a cell and ******,

solubilizes, and is prepared.

[0018] A surfactant is used in order to destroy a cell membrane and nuclear membrane, to take out the intracellular matter and to prepare the solubilized cell. As the example, Nonidet P-40, a triton X-100, deoxycholic acid, CHAPS, etc. are mentioned. As for surfactant concentration, less than [1 w/v%] is desirable.

[0019] A proteolytic enzyme inhibitor is used in order to prevent destroying protein, when the intracellular matter with which a cell membrane and nuclear membrane were destroyed is intermingled. The commercial item with which these proteolytic enzyme inhibitor [like the protease inhibitor cocktail of the mixture and the sigma company of EDTA, a METARO protease inhibitor like EGTA, PMSF, a trypsin inhibitor, a serine protease inhibitor like a chymotrypsin and/or an iodoacetamide, and a cysteine protease inhibitor like E-64 to marketing] whose example of the is was mixed beforehand is mentioned.

[0020] After solubilizing a cell, the filtration using centrifugal separation or a filter etc. removes insoluble matter from the solution of an organization or a cell. Next, in performing the quantum of protein by the approach of this invention, it is desirable to measure the total amount of proteins in the processed tissue or the solution of a cell according to a well-known approach to this contractor. The total amount of proteins is measured considering Cow IgG as a criterion for example, using DC protein kit etc.

[0021] Subsequently, it has one or more wells for the sample which was made to solubilize the organization or cell which measured the total amount of proteins, and was prepared, and pours into the well in the plate which has arranged the hydrophobic porosity film at the pars basilaris ossis occipitalis, and this film is made to carry out solid phase formation of the protein in a sample by drawing in by negative pressure from the hydrophobic porosity film side of a plate.

[0022] what can carry out a hydrophobic bond to protein as hydrophobic porosity film -- specifically, a PVDF (polyvinylidene fluoride) hydrophobic membrane, a nylon (finishing [electric charge processing]) membrane, a nitrocellulose, etc. are mentioned.

[0023] 0.1-10 micrometers of holes of the hydrophobic porosity film of the bottom of a well are 0.1-0.5 micrometers preferably. About 50 to 1000 mmHg, preferably, suction from the hydrophobic porosity film side of a plate is the pressure of about 100 to 300 mmHg, and can be performed for about 5 - 120 seconds. It can choose as magnitude of the bottom of a well, taking total of the area of base of a well, the ease of carrying out of suction by the negative pressure from a film side, etc. into consideration.

[0024] Since protein is held by hydrophobic association between film etc. even if the

hole of the hydrophobic porosity film is larger than protein when carrying out solid phase formation of the protein on the hydrophobic porosity film, protein does not pass the hydrophobic porosity film. In addition, it is desirable to use what performed initialization processing of the immersion to a transfer buffer (48mM tris, 39mM glycine, 20% methanol, SDS, and 80% water) etc. as this hydrophobic porosity film.

[0025] The amount of the sample poured distributively to a well can be easily determined as this contractor in consideration of the class of the amount of the total protein contained in this sample, and protein aimed at obtaining a quantum. under the present circumstances, the sample which contains the protein of an amount smaller than the amount in which solid phase formation is possible on the hydrophobic porosity film of a predetermined area of the bottom of a well -- each -- pouring distributively to a well is desirable. A sample can dilute with a tris buffer, a phosphoric-acid buffer, and a diluent like water, and when the amount of the quantum purpose protein contained in a sample is expected [more / on the hydrophobic porosity film of a predetermined area of the bottom of a well] than the amount in which solid-phase formation is possible, it can adjust and use so that the amount of the total protein contained in the sample poured distributively to a well may turn into below the amount in which the solid-phase formation to per predetermined area of the hydrophobic porosity film is possible.

[0026] For example, the amount of the sample poured distributively using as a sample the sample by which the Hela cell whose total amount of proteins is 1microg/ml was solubilized when the area of base of a well is 2 24mm and the quantum purpose proteins are Cdk2, Cdk4, CyclinE, P16, P21 and P27, C-myc, etc. is about 100microl per well.

[0027] On the other hand, using the sample by which the Hela cell whose total amount of proteins is 0.5microg was solubilized, when the quantum purpose protein is an actin, when the area of base of a well is 2 24mm, it annotates by about 100 lmicro per well. Under the present circumstances, since the amount of the actin contained in the sample by which the Hela cell was solubilized has many contents compared with other quantum purpose proteins, it can dilute this sample with a tris buffer, a phosphoric-acid buffer, and a diluent like water, and can adjust the total amount of proteins.

[0028] As for a plate, it is desirable to have a well one or more. the 1st antibody which has singularity in different protein after making the hydrophobic porosity film of the bottom of a well carry out solid phase formation of the same sample, if prepared one or more -- each -- it is because it pours into a well and the quantum of the protein of

many items can be carried out about one kind of sample. moreover, a different sample -- each -- the 1st antibody which has singularity in the same protein after making the hydrophobic porosity film of the bottom of a well carry out solid phase formation -- each -- it pours into a well and the quantum of one item of the protein can also be carried out about the sample of varieties.

[0029] A concentration gradient may be given to one or more wells of a plate, and the pure article of the protein aimed at obtaining a quantum may be poured distributively to them. For example, in case a well carries out those with six at one train and makes the solid phase formation of the protein aimed at obtaining a quantum carry out there as shown in drawing 1 , the well which poured distributively 0ng (it considers as the background and the purpose protein is not included), 5ng, 12.5ng, 25.0ng, 37.5ng, and the standard sequence 1 included 50 ngs for the quantum purpose protein is prepared. Thus, since it is processed under the same condition as the samples 2-7 by which solid phase formation was carried out into the same plate and by which a quantum should be carried out when solid phase formation of the pure article of the protein aimed at obtaining a quantum is carried out into the same plate, the calibration curve based on the result obtained from the pure article of the protein which has a concentration gradient becomes what has a very high precision, and is desirable (refer to drawing 1).

[0030] in order to avoid combining with an external factor nonspecific at a reaction process with an antibody among the proteins by which solid phase formation was carried out on the hydrophobic porosity film, and producing a measurement error -- arbitration -- a well -- blocking liquid is poured distributively to inside. As for this process, it is desirable to carry out, after making the hydrophobic porosity film carry out solid phase formation of the protein contained in a sample.

[0031] In CdkP16 [2], as for blocking liquid, the quantum purpose protein can use TBS-T (Tris Buffered Saline Tween) and 4%BSA (bovine serum albumin), when 50%Block ace and protein are an actin, Cdk4, CyclinE, P53, P21 and P27, and C-myc. It is made to put and react to a well at a room temperature for 0 - 60 minutes after pouring blocking liquid distributively, and the film is attracted and removed from the hydrophobic porosity film side of a plate by negative pressure as mentioned above after that.

[0032] subsequently, the 1st antibody which is labeled, has a reactant part with an indicator, and has the protein and singularity for the purpose of measurement -- this -- a well -- it pours into inside and is made to combine with the protein for the purpose of measurement With the antibody labeled, the labeled well-known antibody

can be used in the field concerned. The antibody which it was labeled with the indicator fluorescent material by the detail, or was labeled with marker enzyme is meant.

[0033] As an indicator fluorescent material, a fluorescein, a coumarin, eosine, a phenanthroline, a pyrene, a rhodamine, etc. are mentioned. Among those, a fluorescein is desirable. A beta galactosidase, the alkaline phosphatase, and a peroxidase are mentioned as marker enzyme. Among those, a peroxidase is desirable.

[0034] The antibody which has a reactive site with an indicator with the antibody well-known in the field concerned which has a reactive site with an indicator can be mentioned. The isothiocyanate part of FITC reacts with the amino group of an antibody, and combines the reactive site with the antibody in the case of Marker FITC (fluorescein iso thia cyanate), and the indicator of the fluorescein is carried out to this antibody. As an antibody, the antibody of the origins, such as a goat, a rabbit, a rat, Buta, a sheep, and a fowl, can be used.

[0035] each -- pour the 1st antibody into a well, it is made to react at a room temperature for 15 - 30 minutes, and the 1st antibody is combined with the protein for the purpose of measurement. The film is attracted by negative pressure as mentioned above from the hydrophobic porosity film side of a plate after that, and the solution is removed. As for the 1st antibody, it can be desirable for it to be used with the gestalt of a solution and to be used with the gestalt of the solution in the tris hydrochloric-acid buffer solution (pH7.4), and the solution can contain a sodium chloride, and ATP and DTT further. The amount of the 1st antibody contained in a solution is suitably adjusted so that more amounts than the amount of measurement purpose proteins expected that solid phase formation is carried out for every hydrophobic porosity film of the bottom of a well can supply a well in consideration of the amount of the total protein in the sample measured previously.

[0036] Subsequently, washing processing is performed and the unreacted 1st antibody is removed. Specifically a penetrant remover is poured into a well, the film is attracted as mentioned above from the hydrophobic porosity film side of a plate, and a penetrant remover is removed.

[0037] As a penetrant remover, TBS-T (250mM tris, a 150mM sodium chloride, 0.05% Tween 20) etc. can be used. The process of washing is performed two or more times once or more.

[0038] When the 1st antibody without an indicator is used, an indicator is made to act on a reactant part with an indicator, and this antibody is made to label. When using a commercial biotin-ized antibody as the second antibody, specifically, it is possible to

make avidins (for example, FITC indicator avidin, HRP indicator avidin, rhodamine indicator avidin, etc.) with a detectable indicator react with a biotin-ized antibody, and to detect them.

[0039] Subsequently, the amount of the indicator combined with the protein for the purpose of measurement is measured. According to an indicator, the amount of an indicator is measured according to a well-known approach in the field concerned. In a detail, the hydrophobic porosity film is removed from a plate, and the amount of an indicator fixed on this film divided by the well is measured in it.

[0040] More, when the 1st antibody is labeled by the detail with an indicator fluorescent material, in it, the amount of fluorescence from this indicator fluorescent material is measured. An indicator fluorescent material is excited on a certain specific wavelength, and, specifically, fluorescence image-analysis equipment detects. Although the wavelength of light to irradiate changes with indicator fluorescent materials, when an indicator fluorescent material is a fluorescein, it irradiates the wavelength of 488nm and excites it, for example.

[0041] When the 1st antibody is labeled with marker enzyme, a substrate which the detectable matter produces optically by the reaction with this marker enzyme is made to act on this marker enzyme, and the amount of the produced product is measured optically. Optically, the detectable matter can react with marker enzyme, and means the matter which can detect the existence by measuring fluorescence, an absorbance, scattered-light reinforcement, transmitted light reinforcement, etc., for example, coloring matter, such as ECL-plus and TMB (tetramethyl benzine), luciferin, etc. are mentioned by the reaction with marker enzyme. Among those, ECL-plus is desirable. When marker enzyme is a peroxidase, specifically, as for the detectable matter, ECL-plus is optically mentioned by the reaction with marker enzyme. In addition, the substrate of marker enzyme can be suitably chosen according to the marker enzyme to be used.

[0042] Subsequently, the amount of purpose proteins is computed by using the amount of this indicator based on the calibration curve produced beforehand. The amount of the purpose protein contained in the solubilization sample of the organization or the cell by which solid-phase formation was carried out on the hydrophobic porosity film is computable by applying the obtained amount of fluorescence to the calibration curve of the amount of fluorescence which processed similarly the pure article protein of the known amount which produced the measured amount of fluorescence beforehand, and was obtained, and the amount of the protein, when an antibody is labeled with an indicator fluorescent material. When an antibody is

labeled with marker enzyme, the amount of purpose proteins contained in the solubilization sample of the organization or cell by which solid phase formation was carried out on the hydrophobic porosity film can be computed by applying the measured value obtained by the calibration curve which produced beforehand the amount of the product produced by the reaction with this marker enzyme like the above.

[0043] In case the amount of an indicator is measured, when the labeling antibody which has the singularity according to the protein for the purpose of measurement has reacted by the well which differs in a location, since a fluorescence detection machine for them to detect the fluorescence for the same indicator can measure the protein of two or more kinds of purposes by using only one kind of excitation wavelength, it is more desirable.

[0044] The sample which was made to solubilize the following process:(1) organizations or cells, and was furthermore prepared by this invention Have one or more wells and it pours into the well in the plate which has arranged the hydrophobic porosity film at the pars basilaris ossis occipitalis of a well. This film is made to carry out solid phase formation of the protein in a sample by drawing in from the hydrophobic porosity film side of a plate. It pours into inside. (2a) the 1st antibody which has the protein and singularity for the purpose of measurement -- this -- a well -- Make it combine with the protein for the purpose of measurement, perform (3) washing processing, and the unreacted 1st antibody is removed. It pours into inside. (3a) the 2nd antibody which is labeled, has a reactant part with an indicator, and has this 1st antibody and singularity -- this -- a well -- When the 2nd antibody which is not labeled by making it combine with this 1st antibody (4a) is used the amount of the indicator which the indicator was made to act on a reactant part with an indicator, and this antibody was labeled, and was combined with the protein for the purpose of (5) measurement -- measuring -- (6) -- based on the calibration curve produced beforehand, the amount of this indicator is used and the measuring method of the amount of proteins which consists of computing the amount of purpose proteins is offered.

[0045] A reactant part with the same labeling and same indicator as the 1st antibody of the above can be used for the 2nd antibody which is labeled or has a reactant part with an indicator. If the 2nd antibody has singularity in the 1st antibody, also in case a different thing for every protein for the purpose of measurement does not need to be used for it, therefore it will measure the protein of many items, the 2nd common antibody may be used for it. If the number of the 1st antibody is one, the 2nd antibody

which has singularity in the 1st antibody is good at one kind.

[0046] Moreover, this invention offers the approach of diagnosing gastric cancer, colon cancer, a breast cancer, lung cancer, an esophagus cancer, a prostatic cancer, hepatic carcinoma, kidney cancer, vesical cancer, skin carcinoma, a uterine cancer, a brain tumor, a flesh-and-blood kind, or a cancer disease like a bone marrow neoplasm, by the result of the amount of proteins measured by the approach of this invention. For example, the patient may be suffered from gastric cancer or colon cancer if the amount of proteins, such as an actin, Cdk2 and Cdk4, CyclinE, P16, P21 and P27, and C-myc, fluctuates.

[0047] Moreover, the plate 23 with which the approach of this invention has the well 21 which plurality as shown in drawing 2 penetrated, and the liquid supply way 22 was established in the end, The base material with which it had two or more one units which consist of hydrophobic porosity film 24 arranged at the pars basilaris ossis occipitalis of a well, and a plate and the hydrophobic porosity film were prepared respectively possible [balking], The liquid supplied to the well can be suitably performed using the sample measuring device which consists of a suction device 25 for drawing in in the direction of a pars basilaris ossis occipitalis of a well from a liquid supply way.

[0048] As this sample measuring device is the following, in case the approach of this invention is performed, it can specifically be used. the well of a sample measuring device -- a sample solution and preparation liquid are poured into inside, it draws in by negative pressure in the direction of a pars basilaris ossis occipitalis of a well using a suction device, and a liquid is removed from a well. specifically, pass the suction device 27, for example, the suction slot linked to the solid phase waste fluid tubing 26, and the gutter 29 linked to the overflow waste fluid tubing 28 -- being drawn in by negative pressure -- a well -- an inner liquid is removed. Consequently, on the hydrophobic porosity film, solid phase formation of the protein in a sample solution or preparation liquid etc. is carried out. pouring in the solution which contains an antibody in the well of the sample measuring device with which solid phase formation of the protein etc. was carried out on the hydrophobic porosity film of the pars basilaris ossis occipitalis of a well, and leaving it under predetermined conditions -- a well -- an antigen-antibody reaction etc. is advanced in inside. A liquid is removed from a well as mentioned above after a reaction using the suction device of this equipment. moreover, a well -- in case inside is washed, a penetrant remover etc. is poured into two or more wells by the liquid supply way of this equipment, and it removes from a well using the suction device of this equipment. thus -- if the suction

device of this equipment is used -- from two or more wells in a plate -- at once -- a short time -- a liquid -- being removable -- the approach of this invention -- a short time -- and it can carry out easily.

[0049]

[Example] In order to explain the approach of this invention to a detail more, the protocol of an example is shown below. Example 1(approach of invention only using 1st antibody) (refer to drawing 3) 1. In the transfer buffer (48mM tris, 39mM glycine, 20% methanol, 0.1%SDS, and 80% water), it was immersed and the PVDF (poly vinylidene fluoride) membrane was initialized.

2. The plate with which the area of base of one well consists of wells of 15 of five-line three trains of regularity (24mm2) was attached on the initialized PVDF membrane, and it fixed so that the base of a well might consist of PVDF membranes.

[0050] 3. five-line one train of a plate -- each -- the cultured cell (Hela) solution whose total amounts of the protein in TBS which contains 0.001% of NP-40 in a well are 1microg/100microl, and the mixture containing 0.001% of NP-40 of the rabbit IgG antibody of strange-among TBS concentration were poured in 100microl every (sample sequence 8).

4. another five-line one train of the same plate -- each -- the cultured cell (Hela) solution whose total amounts of the protein in TBS which contains 0.001% of NP-40 in a well are 1microg/100microl was poured in 100microl every (negative control sequence 9).

[0051] 5. another five-line one train of the same plate -- each -- the rabbit IgG antibody of 0ng/100microl, 2ng/100microl, 4ng/100microl, 8ng/100microl, and 16ng(s)/100microl was poured in 100microl every all over TBS which contains 0.001% of BSA of NP-40 and 1micro g/100microl in a well. All over TBS where a rabbit IgG antibody contains 0.001% of BSA of NP-40 and 1micro g/100microl, a 1.5M sodium chloride water solution is diluted with the included 250mM tris, and is adjusted to predetermined concentration, and the rabbit IgG antibody of 8ng(s)/100microl was made for the total amount of proteins to be set to 1micro g/100microl (standard sequence 10).

[0052] 6. the negative pressure from the base of the well after impregnation of a predetermined liquid is completed to all the wells in a plate, i.e., the rear face of a membrane, -- it drew in for about 15 seconds by about 200 mHg(s).

7. Subsequently, the penetrant remover (TBS-T:250mM tris, a 1.5M sodium chloride water solution, 1.0% Tween 20) was poured into all the wells in a plate, and it drew in for about 30 seconds by negative pressure abbreviation 500mHg from the base of a

well after that.

8. Blocking liquid (TBS-T, 4%BSA) was poured into all the wells in a plate every [100micro / l], and it put on them gently at the room temperature for about 30 minutes. Then, it drew in for about 15 seconds by negative pressure abbreviation 500mHg from the base of a well, and, subsequently all the wells in a plate were washed like the previous process 7.

[0053] 9. the anti-rabbit antibody (1.5mg [/ml] TBS-T solution of 1/4000 FITC anti rabbit IgG) by which the indicator was carried out to the rabbit IgG antibody by FITC (fluorescence isothiocyanate) combined specifically was poured into all the wells in a plate 100microl every, and it put on them gently at the room temperature for about 30 minutes. Then, it drew in for about 30 seconds by negative pressure abbreviation 500mHg from the base of a well, and, subsequently all the wells in a plate were washed like the previous process 7.

10. After it removed a PVDF membrane from a plate and distilled water washed, it was made to dry at a room temperature for about 15 minutes. then, a PVDF membrane -- a fluorescence reader -- using -- each -- the fluorescence emitted from the marker combined with the protein by which the magnitude corresponding to the base of a well was adsorbed was read with the fluorescence reader.

[0054] 11. The amount of proteins of a sample was computed based on the average fluorescence intensity obtained from the sample sequence 8 (five wells), the average fluorescence intensity obtained from the negative control sequence 9 (five wells), and the fluorescence intensity obtained from the well of each concentration of the SUTAN dirt sequence 10 (five wells). In addition, since IgG protein is the fluorescence intensity obtained although it does not adsorb on the membrane, the average fluorescence intensity obtained from the negative control sequence 9 (five wells) is considered to be background fluorescence, such as autofluorescence which a membrane has.

[0055] Therefore, the following formula:(fluorescence intensity of net sample) = (average fluorescence intensity obtained from the sample sequence) - (average fluorescence intensity obtained from the negative control sequence)

By it being alike and applying, the fluorescence intensity of the net of the sample except background fluorescence is computed.

[0056] Moreover, since the fluorescence intensity obtained among the standard sequences 10 (five wells) at the time of rabbit IgG concentration 0ng/100microl is the fluorescence acquired although solid phase formation of the rabbit IgG protein is not carried out on the membrane, it is considered to be the autofluorescence which a

membrane has, and the background fluorescence by the interaction of BSA which has diluted Rabbit IgG. Therefore, the following formula:(net standard fluorescence intensity) = (fluorescence intensity obtained from the standard sequence) − (fluorescence intensity obtained from standard sequence 0ng/100microl)
By it being alike and applying, the fluorescence intensity of the net of the standard sequence except background fluorescence is computed.

[0057] Since the result of this example 1 was 563.average fluorescence intensity =6 count obtained from the average fluorescence intensity =4061.6 count negative control sequence acquired from the sample sequence, the fluorescence intensity of a net sample is 3498 counts.

[0058] On the other hand, it is as the rabbit IgG concentration which computes an approximate expression from the concentration of IgG and corresponding fluorescence intensity since the result of this example 1 was fluorescence intensity =378 count obtained from rabbit IgG concentration 0ng/100microl obtained from the standard sequence showing the fluorescence intensity of the net of the standard sequence of concentration 0ng / other than 100microl in Table 1.

[0059]

[Table 1]

スタンダード系列の蛍光強度

IgG濃度 [ng／100μl]	2	4	8	16
蛍光強度 [カウント]	367.8	849.6	2316.4	4394.6

[0060] A straight-line approximate expression is computed from the concentration of IgG, and corresponding fluorescence intensity. The result of this example 1 was fluorescence intensity =283.16x (IgG concentration). Therefore, installation of fluorescence intensity 3498 count of a sample computed the IgG concentration of a sample with 12.4ng(s) / the 100microg total protein.

[0061] Example 2(approach of invention using 1st antibody and 2nd antibody) (refer to drawing 4) 1. In the transfer buffer (48mM tris, 39mM glycine, 20% methanol, 0.1%SDS, and 80% water), it was immersed and the PVDF (poly vinylidene fluoride) membrane was initialized.

2. The plate with which the area of base of one well consists of wells of 18 of six-line three trains of regularity (24mm2) was attached on the initialized PVDF membrane, and it fixed so that the base of a well might consist of PVDF membranes.

3. six-line one train of a plate -- each -- the cultured cell (Hela) solution whose total

amounts of the protein in TBS which contains 0.001% of NP-40 in a well are 1microg/100microl was poured in 100microl every (sample sequence 11).

[0062] 4. another six-line two trains of the same plate -- each -- the solution of the pure preparation of the parameter of five kinds of concentration which contains 0ng/100microl all over TBS which contains 0.001% of BSA of NP-40 and 1micro g/100microl in a well was poured in 100microl every. The pure preparation of a parameter is diluted with the 250mM tris which contains a 1.5M sodium chloride water solution all over TBS containing 0.001% of NP-40, and 10microg/10ml BSA, and is adjusted to predetermined concentration, and it was made for the total amount of proteins to be set to 1micro g/100microl (standard sequence 12). Within the same plate, this standard sequence 12 pours in the preparation of the same class, and creates a standard sequence in a plate which is different whenever parameters differ.

[0063] 5. the negative pressure from the base of the well after impregnation of a predetermined liquid is completed to all the wells in a plate, i.e., the rear face of a membrane, -- it drew in for about 15 seconds by about 200 mHg(s).

6. Subsequently, the penetrant remover (TBS-T:250mM tris, a 1.5M sodium chloride water solution, 1.0% Tween 20) was poured into all the wells in a plate, and it drew in for about 30 seconds by negative pressure abbreviation 500mHg from the base of a well after that.

[0064] 7. all the inside of a plate -- all -- blocking liquid (TBS-T, 4%BSA) was poured into the well 100microl every, and it put on it gently at the room temperature for about 30 minutes. Then, it drew in for about 15 seconds by negative pressure abbreviation 500mHg from the base of a well, and, subsequently all the wells in a plate were washed like the previous process 6.

8. the preparation of a parameter poured in the solution of the rabbit antibody (the 1st antibody) specifically combined with the preparation of the parameter corresponding to all the wells in the adsorbed plate 100microl every, and put at the room temperature for about 30 minutes.

[0065]

[Table 2]

測定項目と、対応する測定項目の標品に特異的に結合するウサギ抗体の種類

測定項目	対応する測定項目の標品に特異的に結合するウサギ抗体の種類	溶液の溶媒
Cdk2	rabbit anti-Cdk2 IgG	TBS (5mM DTT,50%グリセロール)
Cdk4	rabbit anti-Cdk4 IgG	TBS (5mM DTT,50%グリセロール)
CyclinE	rabbit anti- CyclinE IgG	TBS (5mM DTT,50%グリセロール)
P16	rabbit anti-P16 IgG	TBS (5mM DTT,50%グリセロール)
P53	rabbit anti-P53 IgG	TBS (5mM DTT,50%グリセロール)
P21	rabbit anti-P21 IgG	TBS (5mM DTT,50%グリセロール)
P27	rabbit anti-P27 IgG	TBS (5mM DTT,50%グリセロール)
C-myc	rabbit anti-C-myc IgG	TBS (5mM DTT,50%グリセロール)

[0066] Then, it drew in for about 15 seconds by negative pressure abbreviation 500mHg from the base of a well, subsequently the same actuation as the previous process 6 was repeated twice, and all the wells in a plate were washed.

9. The biotin-ized anti-rabbit antibody (the 2nd antibody) (1 in TBS-T / 100 anti-rabbit IgG biotiylated solutions which contain BSA 1%) was poured in to the well of all plates. Then, it drew in for about 15 seconds by negative pressure abbreviation 500mHg from the base of a well, subsequently the same actuation as the previous process 6 was repeated twice, and all the wells in a plate were washed.

[0067] 10. all the inside of a plate -- all -- the FITC indicator streptoavidin reagent (1/100 FITC strept avidin) was poured into the well 100microl every, and it put on it gently at the room temperature for about 30 minutes. Then, it drew in for about 15 seconds by negative pressure abbreviation 500mHg from the base of a well, subsequently the same actuation as the previous process 6 was repeated 3 times, and all the wells in a plate were washed.

11. After it removed a PVDF membrane from a plate and distilled water washed, it was made to dry at a room temperature for about 15 minutes. then, a PVDF membrane -- a fluorescence reader -- using -- each -- the fluorescence emitted from the matter which carried out the indicator of the protein by which the magnitude corresponding to the base of a well was adsorbed was read with the fluorescence reader.

[0068] 12. The amount of proteins of a sample was computed based on the fluorescence intensity obtained from the well of each concentration of the average fluorescence intensity obtained from the sample sequence 11 (six wells), and the SUTAN dirt sequence 12 (six wells x2 train). In addition, since the protein of a parameter is the fluorescence intensity obtained although it does not adsorb on the membrane, the average fluorescence intensity obtained from the concentration 0 (two

wells) of a standard sequence is considered to be background fluorescence, such as autofluorescence which a membrane has. Therefore, the following formula:(fluorescence intensity of net sample) = (average fluorescence intensity obtained from the sample sequence) − (average fluorescence intensity obtained from the standard sequence of concentration 0)

By it being alike and applying, the fluorescence intensity of the net of the sample except background fluorescence is computed.

[0069] The fluorescence intensity of the fluorescence intensity obtained from the standard sequence of the preparation of the parameter from which concentration differs, the graph which shows the calibration curve acquired from there, and the measured sample, and the concentration of the computed parameter are shown below.

[0070] (i) Measurement of Cdk2 [Table 3]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

Cdk2（ng／ウエル）	0.00	2.00	5.00	10.00	15.00
蛍光強度（カウント）	1285.59	1635.78	2116.93	3062.92	3735.63

[0071] (ii) Measurement of Cdk4 [Table 4]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

Cdk4（ng／ウエル）	0.00	2.00	5.00	10.00
蛍光強度（カウント）	1450.98	2314.64	3374.60	4776.19

[0072] (iii) Measurement of CyclinE [Table 5]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

CyclinE（ng／ウエル）	0.00	20.00	50.00	100.00	150.00
蛍光強度（カウント）	1920.53	5798.08	13318.06	27332.13	34423.52

[0073] (iv) Measurement of P16 [Table 6]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

P16（ｎｇ／ウエル）	0.00	1.00	2.50	5.00	7.50	10.00
蛍光強度（カウント）	419.68	523.54	764.21	1064.81	1309.83	1615.15

[0074] (v) Measurement of P53 [Table 7]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

P53（ｎｇ／ウエル）	0.00	20.00	50.00	100.00	150.00	200.00
蛍光強度（カウント）	1185.37	3232.07	6548.63	12491.52	19104.03	23137.77

[0075] (vi) Measurement of P21 [Table 8]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

P21（ｎｇ／ウエル）	0.00	20.00	50.00	100.00	150.00	200.00
蛍光強度（カウント）	599.48	882.68	1101.06	1465.83	1966.69	2379.38

[0076] (vii) Measurement of P27 [Table 9]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

P27（ｎｇ／ウエル）	0.00	2.00	5.00	10.00	15.00	20.00
蛍光強度（カウント）	503.99	823.53	1520.01	2254.76	2722.69	3528.19

[0077] (viii) Measurement of C-myc [Table 10]

測定項目の濃度と標品のスタンダード系列から得られた蛍光強度

C-myc（ｎｇ／ウエル）	0.00	2.00	5.00	10.00	15.00	20.00
蛍光強度（カウント）	431.05	917.95	1964.85	3343.28	4687.79	5919.69

[0078]

[Table 11]

測定項目	試料の蛍光強度（カウント）	試料中の量（ｎｇ／ウエル）	試料中の濃度（ｎｇ／１μｇ）
Cdk2	1121.8668	6.641511	6.641510509
Cdk4	413.61338	0.853098	0.853098498
CyclinE	551.37006	1.81718	2.022433168
P16	461.820014	3.762923	3.762923089
P53	266.630028	2.926445	2.926444763
P21	602.132563	63.84379	63.84378535
P27	476.490014	2.49423	2.494230426
C-myc	140.0750042	0.355846	0.410197873

[0079]

[Effect of the Invention] The detection or the quantum of the protein of many items which follow, and the sample which carried out solubilization processing of a cell or the organization is made to stick to the hydrophobic porosity film quantitatively directly, and is performed at a short time and few processes using one kind of specific antibody to the protein for the purpose of detection is possible for this invention.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is drawing showing the standard to the plate used by the approach of this invention, and arrangement of distributive pouring of a sample.

[Drawing 2] It is (a) drawing of longitudinal section and the (b) cross-sectional view showing a suitable sample measuring device to perform the approach of this invention.

[Drawing 3] It is drawing showing the standard to the plate used in the example 1 of this invention, and arrangement of distributive pouring of a sample.

[Drawing 4] It is drawing showing the standard to the plate used in the example 2 of this invention, and arrangement of distributive pouring of a sample.

[Drawing 5] It is drawing showing the calibration curve of Cdk2 obtained by the approach of this invention.

[Drawing 6] It is drawing showing the calibration curve of Cdk4 obtained by the approach of this invention.

[Drawing 7] It is drawing showing the calibration curve of CyclinE obtained by the approach of this invention.

[Drawing 8] It is drawing showing the calibration curve of P16 obtained by the approach of this invention.

[Drawing 9] It is drawing showing the calibration curve of P53 obtained by the

approach of this invention.

[Drawing 10] It is drawing showing the calibration curve of P21 obtained by the approach of this invention.

[Drawing 11] It is drawing showing the calibration curve of P27 obtained by the approach of this invention.

[Drawing 12] It is drawing showing the calibration curve of C-myc obtained by the approach of this invention.

[Description of Notations]

1 Standard Sequence

2 Sample 1

3 Sample 2

4 Sample 3

5 Sample 4

6 Sample 5

7 Sample 6

8 Sample Sequence

9 Negative Control Sequence

10 SUTAN Dirt Sequence

11 Sample Sequence

12 Standard Sequence

21 Well

22 Liquid Supply Way

23 Plate

24 Hydrophobic Porosity Film

25 Suction Device

26 Solid Phase Waste Fluid Tubing

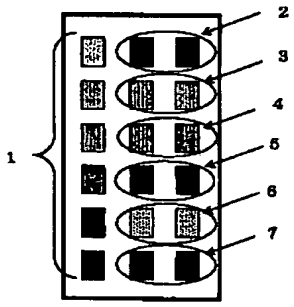
27 Suction Slot

28 Overflow Waste Fluid Tubing

29 Gutter

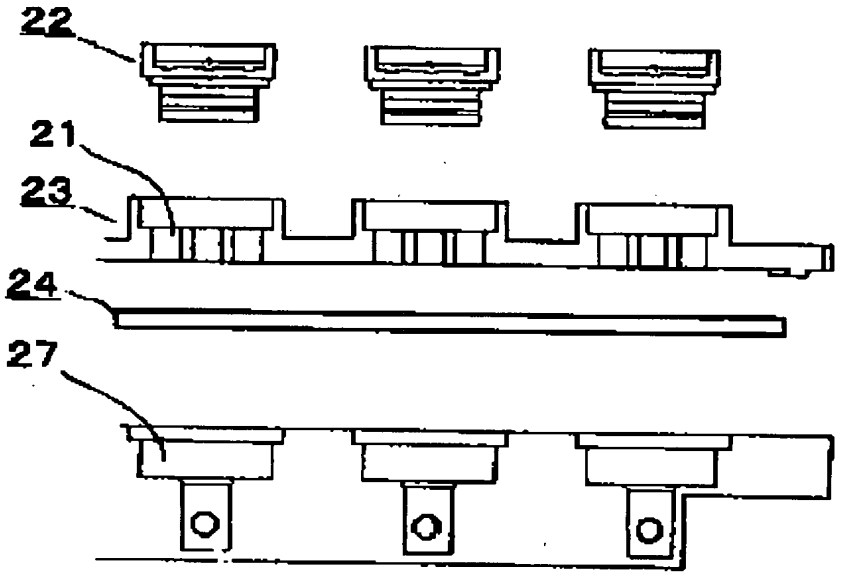
DRAWINGS

[Drawing 1]



[Drawing 2]

(a)



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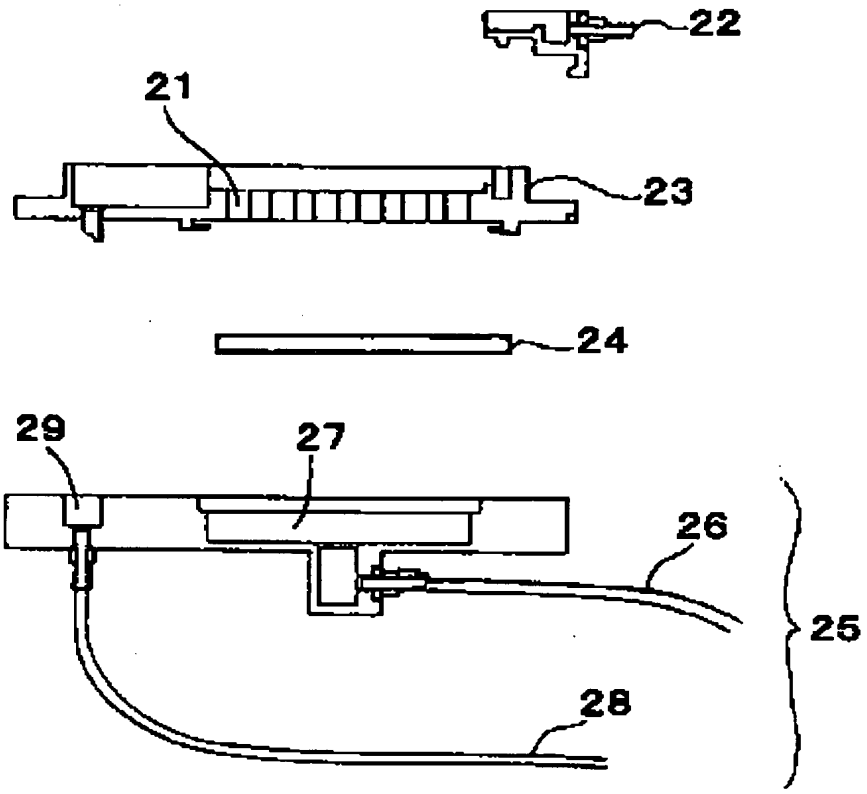
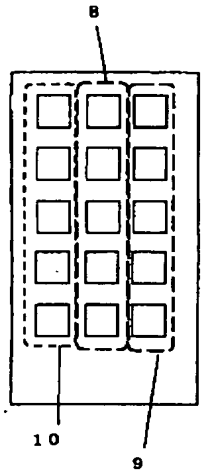
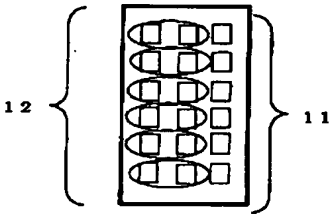


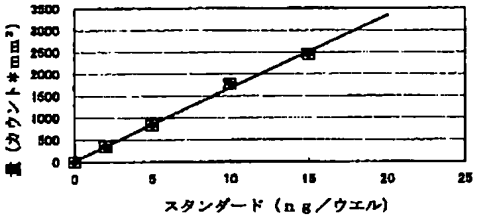
[Drawing 3]



[Drawing 4]



[Drawing 5]



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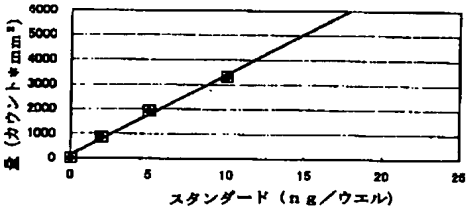
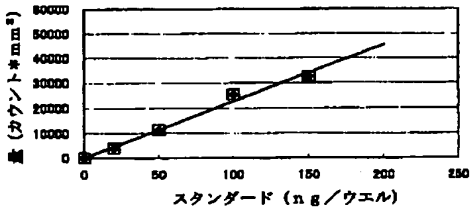
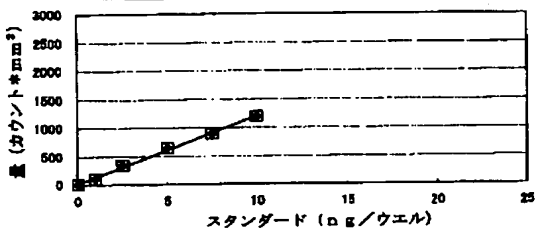


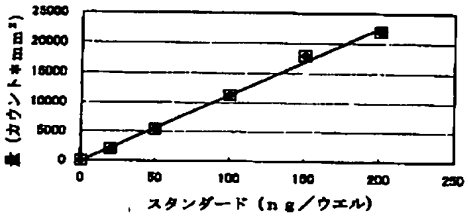
[Drawing 7]



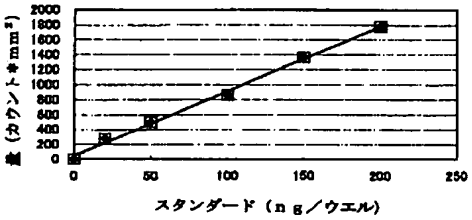
[Drawing 8]



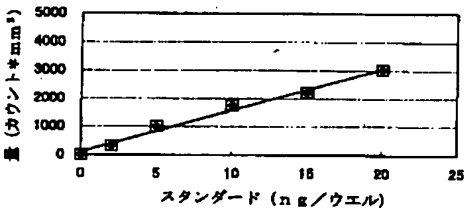
[Drawing 9]



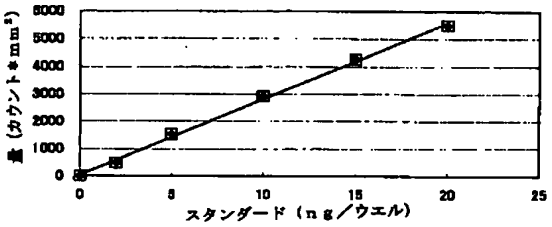
[Drawing 10]



[Drawing 11]



[Drawing 12]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